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Original Article

Locomotor and histological changes in a cuprizone-induced animal model of multiple sclerosis: comparison between alpha-tocopherol and fingolimod

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Abstract

Background and purpose: Fingolimod is a sphingosine 1-phosphate receptor modulator used to treat multiple sclerosis (MS). Alpha-tocopherol (AT) has been found to improve motor function in an animal model of MS. In the present study, the effects of AT and fingolimod on the locomotor function and histological evidence of demyelination were compared in a cuprizone-induced rat model of MS.
Experimental approach: Female Sprague-Dawley rats (8 weeks) were fed with 0.2% (w/w) cuprizone diet for 5 weeks followed by intraperitoneal injections of fingolimod (3 mg/Kg; group F, n = 10) and alpha-tocopherol (100 mg/Kg; group A, n = 10). Vehicle-treated rats (group V, n = 10) were treated intraperitoneally with 1% ethanol in saline on weeks 6 and 7. Open field and beam walking tests were carried out every 10 days. The mean area of demyelination in the corpus callosum was quantified using Luxol fast blue stained histological sections of the forebrain.
Findings/Results: The mean speed of movement was increased by 54% and 50% in groups F and A compared to group V. Total distance moved was increased by 61% and 52.7% in groups F and A compared to group V. Mean time to walk the beam was reduced in group A by 52% compared to group V. Mean frequency of crossing lines from the inner squares to outer squares was reduced in groups A and F compared to group V. Mean area of demyelination in corpus callosum showed 62% reduction in group A compared to group V.
Conclusion and implications: Both fingolimod and AT treatments improved the locomotor function. However, AT treatment reduced the areas of demyelination in higher proportion and improved motor coordination and exploratory behavior.

Keywords: Alpha-tocopherol; Demyelination; Fingolimod; Cuprizone; Multiple sclerosis.

INTRODUCTION

Demyelination in the central nervous system (CNS) induced by the toxins, which are injurious to the oligodendrocytes and myelin, turns to remyelination following the withdrawal of the toxins (1). Cuprizone (CPZ)-induced demyelination model has been widely tested in *in vivo* studies as it exhibits demyelination behaviours similar to those observed in patients with multiple sclerosis (MS). The model provides reproducible demyelination and spontaneous remyelination processes (2). It is documented that after acute CPZ-induced demyelination, the potency of a pharmaceutical compound can be tested to find whether it can accelerate the ongoing remyelination process. It is recently established that following the cessation of CPZ treatment, initial recovery of locomotor performance is found which declines again in remyelinated animals due to brain atrophy associated with axonal loss in the corpus callosum (3).

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Demyelination in MS is preceded by the transfer of the CD4+ anti myelin lymphocytes. In addition, the cytotoxic CD8+ T cells play a crucial role in demyelination. Antigen-presenting cells are the critical players in perpetuating the inflammatory milieu (4). Destruction of the myelin is responsible for the clinical signs of MS manifested as gait disturbance, impaired coordination, and sensory loss (5). Sphingosine 1-phosphate (S1P) receptors are differentially expressed by the immune and CNS cell types. T and B cells mainly express the S1P receptors (6). Fingolimod (FTY720), a small molecule that acts as an S1P receptor modulator, was approved by the FDA for the treatment of MS. Fingolimod blocks egress of effector T cells from peripheral lymphoid tissues to the CNS (7).

Vitamin E is a natural antioxidant, and it suppresses the peroxidation of membrane lipids by scavenging oxygen and superoxide anion radicals. Alpha-tocopherol (AT) and tocotrienol are the main components of vitamin E. Synthetic forms of AT are known as DL-AT acetate, chemically known as all-rac-2, 5, 7, 8-tetramethyl-2-(4, 8, 12-trimethyltridecyl)-3,4-dihydro-2H-1 benzopyran-6-yl acetate (8). AT has been proven to cross the blood-brain barrier to produce the therapeutic effect in the CNS due to its lipophilic property (9). Open field test (OFT) is a type of neuro-behavioral test to measure locomotor activity, exploratory behaviour, and anxiety-related behaviour in rodents (10). Beam walking test (BWT) is used to assess sensorimotor balance and coordination ability (11).

In our previous study, AT has been found to increase the average speed of walking and reduce the time taken to traverse the beam in the CPZ-induced rat model of MS. The regenerative effect of AT was also proven by its ability to reduce the areas of demyelination in the corpus callosum (12). In view of emerging evidence of AT as a therapeutic agent in MS, there is a need to compare it with an approved drug such as fingolimod. The present study aimed at comparing the effects of a synthetic form of AT with the similar effects of fingolimod HCl salt on the locomotion and histological evidence of demyelination in the CPZ-induced rat model of MS.

MATERIALS AND METHODS

Experimental process

Female Sprague-Dawley rats, 8 weeks old (200-220 g), were divided into three groups, 10 each. In each cage, two rats were housed with automatic temperature control and a 12/12-h light/dark cycle. Rats in all groups were fed with 0.2% (w/w) CPZ-impregnated chow (Envigo, USA) for the first 5 weeks. During weeks 6 and 7, CPZ was withdrawn from the rats' diet and rats were fed with normal lab chow. During these two weeks, all three groups received daily intraperitoneal (IP) injections. Control (group V) group of rats received 1% ethanol in normal saline IP. Group A received 100 mg/Kg AT in 1% ethanol in normal saline (13) and group F received 3 mg/Kg fingolimod HCl salt in 1% ethanol in normal saline (14,15). Water-miscible DL-AT was purchased from Merck, Malaysia, and fingolimod HCL from Toronto Research Chemicals (TRC), Canada. Animal experiments were conducted according to the principles stated in the guidebook of the Laboratory Animal Care and Use Committee (ACUC) of the University. Ethical approval was taken from the University Joint Committee on Research [Ethics No. BP I-02/2019 (03)].

OFT and BWT

The OFT was conducted to measure the locomotor activity and anxiety-related behaviour of the rats. The protocol described by Seibenhener and Wooten was followed in this study with slight modifications (10). The open box was 30 cm (L) × 30 cm (W) × 60 cm (H) and was evenly illuminated. The base was coloured black with a marked inner zone of 30 cm × 30 cm. The rats were placed at the centre of the arena and the movements of the rats were captured for 10 min using a phone camera (Huawei Nova 3i, 1080p) above the arena. The average speed (m/s) and the total distance moved (m) were tracked and analysed by the ANY-Maze Video Tracking Software Version 6.3 from the captured video (16). Rearing frequency and the frequency of line crossing from inner to outer squares were calculated manually from the video. Rearing is a stereotyped behaviour where the rat stands on its hind legs and lifts the forelegs upwards against the walls. In the OFT, restraint stress was found to reduce the rearing frequency and

an increase in rearing frequency was interpreted as the increased exploratory activity or reduced anxiety (17). The average values for these parameters were calculated per group. The beam walking apparatus was elevated 30 cm above the ground level and 122 cm in length. The study followed the protocol for BWT as described by Luong *et al.* in this experiment (11). Rats were trained to travel across a narrow beam (width 2.5 cm, length 100 cm). Each rat was placed on one side of the beam and a black box with straw bedding was placed on the other side. The time taken to reach a length of 80 cm was determined. The mean values of three consecutive data taken 10 min apart were calculated. OFT and BWT were performed every 10 days for a total of 5 times (three times during weeks 1-5 and 2-times during weeks 6-7 having AT/fingolimod/vehicle treatment) during the experiment. BWT was employed to assess motor coordination (18). Following the protocol of our previous study, the average speed, total distance moved, and BWT were classified under locomotor ability. The frequency of line crossing from inner to outer squares and rearing frequency were classified under exploratory activity (19).

Histological and histomorphometric studies

At the end of week 7, rats were anesthetized with ketamine-xylazine mixture followed by intracardiac perfusion with 4% paraformaldehyde. Following completion of the perfusion, the rats were killed by cervical dislocation. Brains were post-fixed overnight followed by dissection of the forebrain area between optic chiasma and infundibulum. Following paraffin embedding, eight-micron coronal serial sections were stained with Luxol fast blue (LFB). LFB is a histochemical stain for myelin. Myelinated areas were stained dark blue, and the demyelinated areas remained pale. Six rats in each group V, A, and F were used for quantitative analysis. Five randomly selected LFB-stained slides (every 27th section between optic chiasma and infundibulum) in each rat were used (20). Nikon NIS-elements software was used to quantify the areas of demyelination at four random areas of corpus callosum from photomicrographs taken with a bright-field compound Nikon microscope YS 100.

Statistical analysis

For descriptive statistics, mean ± SEM values were calculated from all OFT parameters and BWT data. Mean ± SEM values of the parameters were subjected to One-way ANOVA analysis using SPSS 25 to find out significant ($P < 0.05$) difference in the values between the treatment groups. Post-hoc Bonferroni was used to find out the inter-group differences. Quantitative data of mean ± SEM area of demyelination was subjected to One-way ANOVA analysis.

RESULTS

Changes in the locomotor activity

The mean body weight of the rats in all the three groups was observed to be steadily increasing from week 1 to 5, in accordance with the increasing food intake. There was no significant change in the body weight and food intake during and after the CPZ-impregnated chow diet period. (Fig. 1).

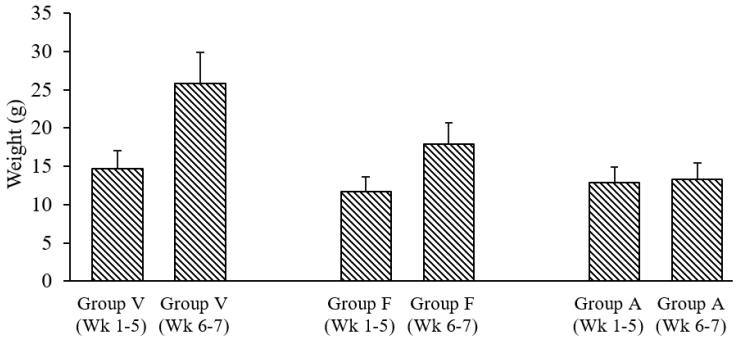

Fig. 1. Consumption of rat pellets in different groups. Vehicle-treated group (V), fingolimod group (F), and alpha-tocopherol group (A) received rat pellet impregnated with 0.2% cuprizone (w/w) during weeks 1-5. The mean amount of rat pellet consumption is similar in the three groups. In weeks 6-7, all groups received normal rat pellets without cuprizone. Data are presented as mean ± SEM.

The average speed of movement in OFT of the CPZ-treated rats in groups F and A, during week 1 to week 5, was reduced by 11.7% and 20%, respectively compared to group V. During withdrawal of CPZ (weeks 6-7), fingolimod treatment increased the average speed by 54% in group F compared to the average speed in vehicle-treated group V ($P < 0.05$). AT treatment in group A showed an increase of 50% in the average speed during similar periods compared to group V ($P < 0.05$) (Fig. 2A). Total distance moved in OFT was reduced in vehicle-treated group V following exposure to CPZ (weeks 6-7) compared to the distance moved during CPZ treatment (weeks 1-5). During withdrawal of CPZ (weeks 6-7) the mean total distance moved in cm increased significantly ($P < 0.05$) in fingolimod-treated group F and AT-treated group A compared to the distance moved in vehicle-treated group V. Mean total distance moved increased by 61% in group F and 52.7% in group A compared to the mean total distance moved in vehicle-treated group V (Fig. 2B). In the BWT, during withdrawal of CPZ (weeks 6-7), the meantime to walk the 80 cm distance of the beam was reduced in the fingolimod-treated group F by 28% compared to the similar time in vehicle-treated group V. In AT-treated group A, mean time to walk the 80 cm distance during withdrawal of CPZ (week6-7), was reduced by 52% compared to the mean time in vehicle-treated group V ($P < 0.05$). There was a significant difference ($P < 0.05$) in mean time taken to traverse the beam only between vehicle-treated group V (9.9 ± 0.8 s) and AT-treated group A (4.7 ± 0.8 s) (Fig. 2C).

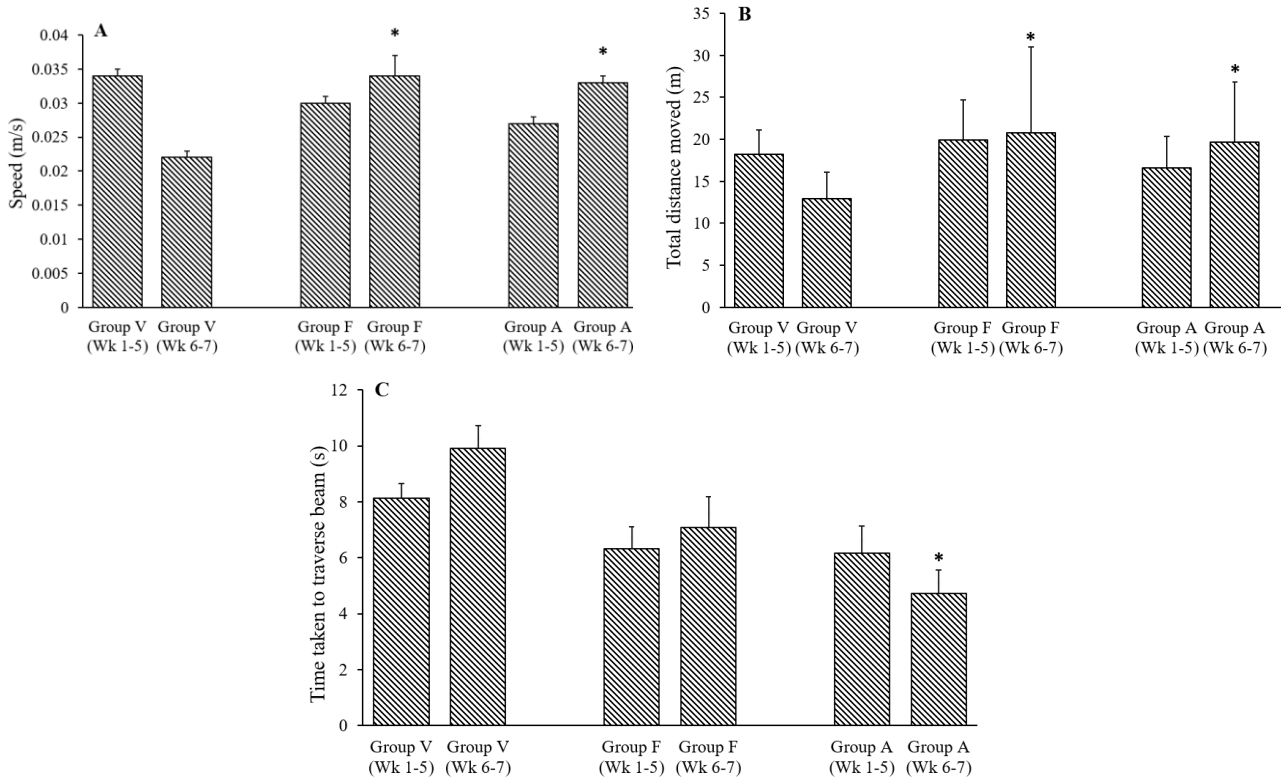

Fig. 2. Effects of fingolimod (group F) and alpha-tocopherol (group A) on (A) average speed, (B) total distance moved, and (C) time taken to traverse the beam in open field test compared to the vehicle-treated group (V). Rats in all groups were fed with 0.2% (w/w) cuprizone-impregnated chow for the first 5 weeks. During weeks 6 and 7, cuprizone was withdrawn from the rats' diet and rats were fed with normal lab chow. Values are mean ± SEM; n = 10. *$P < 0.05$ Indicates significant difference compared to respective group V.

Changes in the exploratory behaviour

Thigmotaxis or anxiety-like behaviour allows the rats to stay closer to the wall of the open field. The mean frequency of crossing the lines from the inner squares to outer squares in OFT, of the CPZ-treated rats in groups F and A during week 1 to week 5 did not show a significant difference compared to the group V. Fingolimod treatment and AT treatment reduced the tendency in the rat-groups to move towards the outer wall of the open-field. During withdrawal of CPZ (weeks 6-7), fingolimod treatment decreased the frequency of line crossing from inner to outer squares by 32% in group F compared to the similar frequency in vehicle-treated group V ($P < 0.05$). AT treatment in group A showed a decrease of 22% in the similar line crossing frequency during similar periods compared to group V ($P < 0.05$) (Fig. 3A). During withdrawal of CPZ (weeks 6-7), the mean frequency of rearing in OFT, increased in fingolimod-treated group F and AT-treated group A compared to the vehicle-treated group V. The rearing frequency observed in 10 min increased by 12.9% in group F and 35% in group A compared to the frequency in group V. Although, AT increased the rearing frequency, the increase was not statistically significant (Fig. 3B)

Histological changes in the corpus callosum

The myelinated white fibre of the corpus callosum in the coronal sections of the forebrain of the rats was stained with LFB. The myelinated areas were stained dark blue. The demyelination was observed in the median and para-median areas of the corpus callosum, by the appearance of band-shaped pale areas in vehicle-treated group V (Fig. 4A). In fingolimod-treated group F, the bluish-stained myelinated fibres appeared to be restored in the median areas of the corpus callosum. However, pale patches of demyelination were observed over the para-median areas (Fig. 4B). In AT-treated group A, the corpus callosum showed bluish-stained myelinated fibres throughout its course except for a small pale demyelinated area over the right para-median area (Fig. 4C).

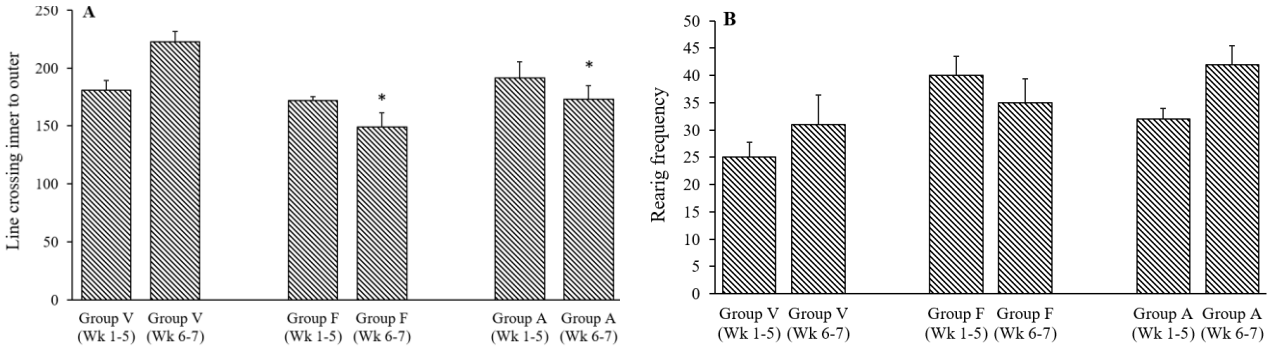

Fig. 3. Effects of fingolimod (group F) and alpha-tocopherol (group A) on (A) frequency of line crossing inner to outer (in 10 min) and (B) rearing frequency (in 10 min) in open field test compared to the vehicle-treated group (V). Rats in all groups were fed with 0.2% (w/w) cuprizone-impregnated chow for the first 5 weeks. During weeks 6 and 7, cuprizone was withdrawn from the rats' diet and rats were fed with normal lab chow. Values are mean ± SEM; n = 10. **P* < 0.05 Indicates significant difference compared to group V.

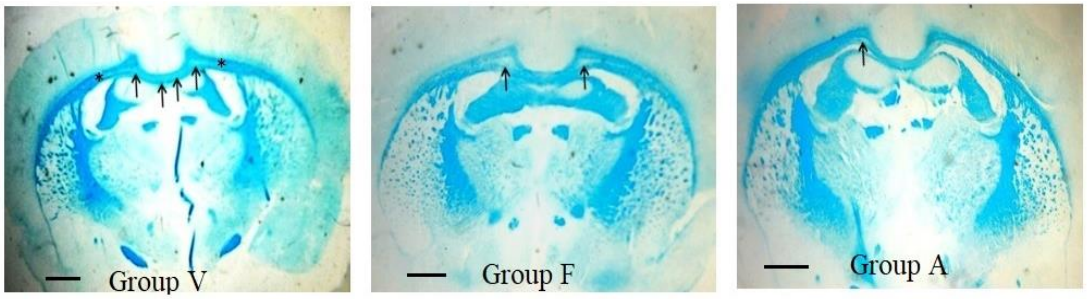

Fig. 4. Luxol fast blue stained histological sections of the forebrain. Brains were collected, at the end of week 7, after vehicle treatment (group V), fingolimod treatment (group F), and alpha-tocopherol treatment (group A). Rats in all groups were fed with 0.2% (w/w) cuprizone-impregnated chow for the first 5 weeks. During weeks 6 and 7, cuprizone was withdrawn from the rats' diet and rats were fed with normal lab chow. * Shows corpus callosum myelinated area; arrows show areas of demyelination. Magnification: ×5.

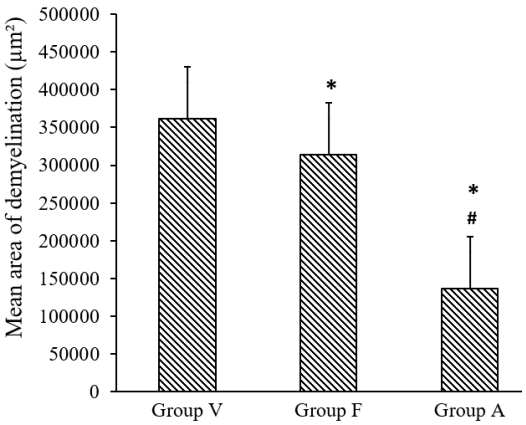

Fig. 5. Effects of vehicle treatment (group V), fingolimod treatment (group F), and alpha-tocopherol treatment (group A) on measurement of mean area of demyelination of corpus callosum (median and para-median part) in luxol fast blue-stained histological sections of the forebrain. Values are mean ± SEM; n = 6. **P* < 0.05 Indicates significant difference compared to group V and #*P*< 0.05 versus group F.

Quantitative changes in the area of demyelination in the corpus callosum

The LFB-stained coronal sections of the iso-cortex from groups V, F, and A were subjected to quantitative analysis. Vehicle-treated group V showed a high mean area of demyelination (361772 square micron) in the corpus callosum. Group F, which received fingolimod, during withdrawal of CPZ, showed a 13% reduction in the mean area of demyelination in the corpus callosum compared to group V ($P < 0.05$). AT treatment reduced the mean area of demyelination in the corpus callosum by 62% compared to the vehicle-treated group V ($P < 0.05$). Both AT-treated and fingolimod-treated groups showed a significant decrease in the demyelinated area in the corpus callosum compared to the vehicle-treated group. However, post-hoc Bonferroni test showed a significant difference in the mean area of demyelination between group A (136853 ± 10581.7 μ^2) and group F (314144 ± 7810.4 μ^2) (Fig. 5).

DISCUSSION

Both fingolimod and AT treatments increased the mean values of the average speed of movement and total distance moved in OFT during the CPZ-withdrawal period. The results showed that both the agents enhanced the improvement in locomotor function recovery as compared to reduced locomotion in the vehicle-treated group. The descriptive analysis of the degree of improvement showed that fingolimod increased the locomotor ability similar to the AT. It increased the average speed by 54% and the total distance moved by 62% in comparison to the AT-induced increase of 50% in the average speed and 52.7% in the total distance moved. The demyelination in the corpus callosum induced by the CPZ has been proven to be due to the apoptosis of the oligodendrocytes brought about by the copper chelator function of the CPZ and subsequent damage to the mitochondria (21,22). Reduced locomotion in the vehicle-treated group of rats was due to the altered regulatory function of the iso-cortex associated with the demyelination of the corpus callosum (23). Remyelination and recovery of the locomotor function require proliferation and migration of the oligodendrocyte precursor cells (OPCs) to the areas of demyelination. The OPC shows higher level of S1P1 gene expression and lower levels of S1P3 and S1P5 genes expression. Hence differentiation and proliferation of OPCs were stimulated in the fingolimod-treated rats due to the S1P modulatory function of the fingolimod (24). AT-treated group A showed improved motor coordination evidenced by the significant decrease in the beam walking time compared to the vehicle-treated group. The reduction in the time taken to traverse the beam was 52% in the AT-treated group compared to 28% reduction in the fingolimod-treated group. AT was found to increase the activity of the limiting enzymes in the monoamine synthesis and increase the level of serotonin, dopamine, and noradrenaline in the hippocampus and striatum, the brain regions actively regulating memory and motor coordination (18).

In OFT, thigmotaxis is quantified by observing the proportion of time that was spent by the animals close to the wall of the box. In this study, when the frequency of line crossing from inner to outer squares of the box was observed, fingolimod-treated rats showed 32% reduction and AT-treated rats showed a 22% reduction in the frequency of line crossing compared to vehicle-treated group V rats and the changes were statistically significant. In an experimental autoimmune encephalomyelitis (EAE) model of MS, prophylactic use of

fingolimod reduced the anxiety-related behaviour in the fingolimod-treated mice compared to fingolimod-untreated mice. Thigmotaxis of fingolimod-treated EAE mice was significantly reduced in 18 days post-immunization compared to the untreated EAE mice (25). Fingolimod was found to modulate the neurochemical transmission of glutamate by acting presynaptically in both animals and patients suffering from demyelinating disorder (26). In a zebrafish model of caffeine-related anxiety-like behaviour, AT in a dose of 1 mg/kg significantly reduced the frequency of thigmotaxis. AT treatment reduced the lipid peroxidation and showed anxiolytic effects by preventing the oxidation of serotonin (27). Rearing is a voluntary act by the rats to map the environment and is essential for their survival. Rearing deficit may happen when there is a failure in spatial memory and novelty detection is impaired. Brainwaves of different frequencies are associated with different cognitive states. Theta frequency is associated with the subconscious mind and reflects activity from the limbic system and hippocampal regions. A study which recorded electrophysiological changes during the rearing in OFT observed increased-theta frequency during the rearing (28). In our study, AT treatment increased rearing frequency in CTZ-withdrawn rats by 35% in group A, compared to vehicle-treated group V. This finding is similar to our previous study, in which AT treatment for 2 weeks increased rearing frequency by 26.1% (12). AT prevented superoxide free radicals from attacking the myelin (29). The resultant metabolic effect caused a potential increase in rearing frequency due to reduction in the stress-induced anxiety.

Qualitative analysis showed that 3 mg/Kg fingolimod hydrochloride treatment for two weeks was not able to remyelinate all the areas of the corpus callosum which were observed to be demyelinated in the vehicle-treated group during the CTZ-withdrawal period. The reduction in the mean area of demyelination was quantified to be 13% compared to the vehicle-treated group. Previous studies found contradictory evidence on the remyelinating effects of fingolimod. In a CPZ-induced model of demyelination, 1 mg/Kg of fingolimod given for two weeks, after 5 weeks of CPZ-treatment, showed no effect on the remyelination. However, a two-fold increase in the OPCs in the corpus callosum was observed (30). In another study, which used a similar model of demyelination, 0.3 mg/Kg of fingolimod treatment modestly accelerated myelin recovery after acute CPZ-induced demyelination but failed to remyelinate following 12 weeks of chronic CPZ treatment (31). A group of rats treated with 100 mg/Kg of AT for two weeks during the CPZ-withdrawal phase, showed remyelination of the corpus callosum in all the areas except a small paramedian area. During CPZ-treatment oligodendrocytes in the myelinated fibres of corpus callosum start to undergo apoptosis. During withdrawal of CPZ, the proliferation of OPCs in the subventricular zone may result at the beginning of a remyelination process (32). As compared to 13% reduction in the mean area of demyelination by fingolimod treatment, AT treatment reduced the mean area of demyelination in the corpus callosum by 62% compared to the vehicle-treated group V. It was suggested that the remyelinating properties of AT were due to its ability to promote the maturation of OPCs by inhibiting the Notch signaling pathway (32).

Both fingolimod hydrochloride and synthetic AT improved locomotor function in CPZ-withdrawn rats in the parameters of average speed and total distance moved in OFT. AT treatment improved motor coordination better than fingolimod as evidenced by a significant reduction in the time to traverse the beam in CPZ-withdrawn rats. AT treatment increased the rearing frequency in CPZ-withdrawn rats. When compared with the fingolimod-treated group, the AT-treated group showed a significant reduction in the area of demyelination in the corpus callosum of CPZ-withdrawn rats. As compared to conventional MS therapeutic practices of using oral forms of fingolimod and synthetic AT, the present study had a limitation of using IP injections. The crucial first step in remyelination is to populate an area of demyelination with sufficient numbers of OPCs. It has been reported that acute axonal damage in experimental models is more likely to be reversible (31,33). The notch signaling pathway plays an important role in myelination. The notch receptors maintain

OPCs at an immature stage and inhibit their differentiation. AT metabolites inhibit Notch signaling and reverse the inhibition of differentiation of OPCs (32). Fingolimod has been reported to reduce the severity of the disease in the relapsing-remitting type of MS by multiple mechanisms which included sequestering lymphocytes, downregulating inflammatory genes, and vascular adhesion molecules, and thus preventing the breach of the blood-brain-barrier. Its S1P receptor modulation mechanism directly regulates the neurons, oligodendrocytes, and astrocytes (34). However, its remyelinating effect in various animal models of MS has been debated due to the difference in the drug delivery route and treatment procedure (35).

CONCLUSION

This study compared the effect of fingolimod (FTY720) and AT in the locomotor function, exploratory behaviour, and histological evidence of the remyelination in the CPZ-induced animal model of MS. Both fingolimod and AT treatment improved the locomotor function in CPZ-withdrawn rats. However, AT treatment reduced the areas of demyelination in higher proportion and improved motor coordination and exploratory behavior.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Author's contribution

N.K. Mitra designed the project, developed the experimental protocol, secured the grant, submitted the project for ethical approval, and conceptualized the manuscript. N. Singh and N.A.N.B. Wadingasafi carried out the experiment, collected and analyzed the data. N. Singh wrote a part of the manuscript. N.K. Mitra and J. Chellian analyzed the final data. All authors reviewed the manuscript. The final version of the manuscript was approved by all authors.

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